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From: Wessendorf,Teresa
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1. Proceedings of the 45th ASMS conference onMass spectrometry and allied Topics, Palm Springs, June 1-5, 1997, p. 907, Siegel et al
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3. Protein Science, 3, 81, (1994), Hutchens et al
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A Rapid Method for Screening Low Molecular Weight Compounds Non-Covalently Bound to Proteins Using Size Exclusion and Mass Spectrometry Applied to Inhibitors of Human Cytomegalovirus Protease

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INTRODUCTION

A property of a useful drug candidate is the ability to form a tightly bound non-covalent complex with its target protein. Using the model system of human cytomegalovirus protease (CMVP), a simple, reliable and rapid method was developed for identifying low molecular weight inhibitors of CMVP which bind non-covalently to the enzyme. The technique utilizes size exclusion GPC spin columns and/or ultrafiltration devices (microconcentrators) for isolating non-covalently bound inhibitor-protease complexes prepared under native conditions, which are then introduced under denaturing conditions into an ESI mass spectrometer, for monitoring and quantitating the individual components of inhibitor and protease. The sample preparation, isolation and detection steps are performed and optimized individually. The methodology is simple to apply and rapid to implement, and allows the characterization of specific and non-specific binding of low molecular weight molecules to protease and the quantitation of the molar ratio of inhibitor to protease in the complex.

EXPERIMENTAL METHOD

Proteases: Wild type CMVP (MW 28,040.6) and mutants A144L (MW 28,082.8), A144D/C87A/C138A/C161A (MW 27,956.7), S132A (MW 28,024.6) and E122V/A144G (MW 27,996.6) were used in the studies. Inhibitors: The inhibitors of CMVP used in these studies are a peptidic difluoroketone, DFK (MW 988.5) (1), two peptidic trifluoromethylketones, TFMK-1 (MW 545) (2) and TFMK-2 (MW 465) (3), and a dibromo quinazoline, DBQ (MW 489) (4). Sample Preparation: CMVP (60 μM) was incubated with a known molar excess of inhibitors in 10mM ammonium acetate (pH 7.5) for 1 hr at 37 °C. The samples were then assayed by size exclusion methods. Gel Permeation Chromatography (GPC) (Spin Columns): GPC spin columns were prepared by filling 1 mL disposable polypropylene syringes (3 mm i.d.) with Sephadex G-25 resin (Pharmacia). The column was centrifuged at 900 x g and the filtrate analyzed. The resin traps molecules <3,000 Da and elutes proteins. Ultrafiltration: Ultrafiltration microconcentrators (3,000 Da cut-off, Amicon Microcon-3, Beverly, MA) were centrifuged at 14,000 x g for 10 minutes. After centrifugation, the filtrate contains material <3,000 Da and the retentate contains material >3,000 Da, such as CMVP or CMVP bound to inhibitor. Mass Spectrometry: Electrospray ionization mass spectra were obtained with a Micromass Quattro triple quadrupole mass spectrometer equipped with a Micromass electrospray source, rf hexapole lens and Megaflow gas nebulizer probe.

RESULTS and DISCUSSION

1) Rapid Screening Size Exclusion-Mass Spectral Assay for Non-Covalently Bound Complexes

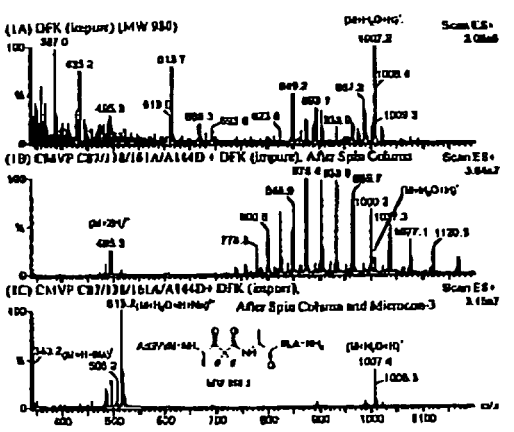
An impure sample of DFK (MW 988.5) (1) (see ESI mass spectrum Figure 1a) was incubated with CMVP A144D/C87A/C138A/C161A in a molar ratio of CMVP:DFK of 1:~10. The resulting mixture was transferred to a GPC spin column and the eluate was analyzed by ESI/MS. As illustrated in Figure 1b, the ESI mass spectrum of the eluate consists of a series of multiply charged peaks related to CMVP in the m/z region of 700-1200 and a series of peaks related to DFK (1) at m/z 1007.4, 495.3 and 486.7 corresponding to $(M+H_2O+H)^{1+}$, $(M+2H)^{2+}$ and $(M+2H-H_2O)^{2+}$, respectively. Note that components corresponding to (1) and the hydrated form of (1) eluted from the spin column together with CMVP demonstrating non-covalent binding of the compounds to CMVP, otherwise, only CMVP would have eluted from the spin column. As a control, DFK (1) alone, at the same concentration used in the incubation experiment, was passed through the spin column, and all peaks corresponding to DFK (1) were absent. Note also that all the minor impurities present in the original DFK (1) sample (Figure 1a) are absent (Figure 1b), indicating that they did not specifically bind to CMVP. Thus, this method for characterizing non-covalent binding is applicable for the analysis of mixtures of compounds; non-covalently bound inhibitors will be selectively coeluted with CMVP while other unbound low molecular weight components will be trapped by the GPC spin column resin. (Similar results were obtained when using a microconcentrator and analyzing by ESI/MS the retentate of the incubated mixture.)

The spin column eluate was next placed into a microconcentrator with a denaturing solution of 3% acetic acid in 1:1 acetonitrile:water. The filtrate was collected and analyzed by ESI/MS (Figure 1c). Note the absence in the mass spectrum of the ion distribution corresponding to the CMVP and the presence of singly, doubly and triply charged peaks corresponding to (1) and the hydrated form of (1).

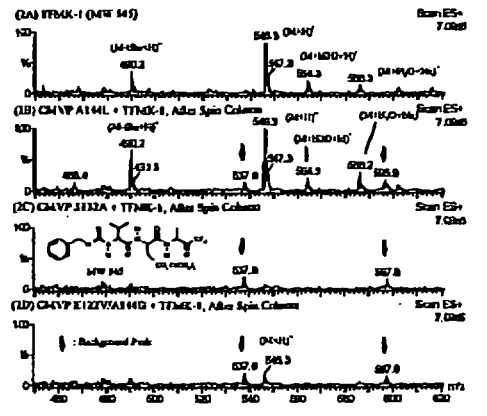
2) Specificity of Non-Covalently Bound Complexes

To examine specificity of binding of compounds to CMVP, two enzymatically inactive mutants of the protease were used. CMVP S132A contains alanine substituted for serine at amino acid 132; this serine is the active site nucleophile which plays a key role in catalysis by CMVP and is expected to be essential for CMVP to bind tightly at the carbonyl carbon of TFMK-1. CMVP E122V lacks the glutamic acid residue which forms a salt bridge in the wild type CMVP; this mutation probably disrupts the normal conformation of the protease. The ESI mass spectrum for inhibitor TFMK-1 (MW 545) (2) (Figure 2a) exhibits the characteristic molecular ions $(M+H)^{1+}$, $(M+H_2O+H)^{1+}$, $(M+H_2O+Na)^{1+}$ and $(M+H_2O+K)^{1+}$ at m/z 546.2, 564.2, 586.2 and 602.1, respectively, as well one fragment ion $(M-C(CH_3)_3+2H)^{1+}$ at m/z 490.1. The ESI mass spectra of the spin column eluates of TFMK-1 incubated with CMVP's A144L (wild type), S132A and E122V/A144G, each prepared at a molar ratio of CMVP:TFMK-1 of 1:40, are illustrated in Figures 2b, 2c and 2d, respectively. TFMK-1 coelutes with CMVP A144L (in a CMVP:TFMK-1 molar ratio of 1:1), does not coelute with CMVP S132A and essentially does not coelute with CMVP E122V/A144G (a molar ratio of CMVP:TFMK-1 of 1:<0.05 was recovered). These coelution results are consistent with the requirement of enzymatically active protease for binding to TFMK-1, strongly suggesting that the binding of this compound to CMVP is specific.

Rapid Screening Mass Spectral Assay
Using Gel Permeation Spin Column / Membrane Filtration



ESI Mass Spectra: Reaction of CMVP With TFMK-1
Demonstration of Coeluted Non-Covalently Bound Drug



3) Competition Study of Inhibitor Mixture with CMVP

A mixture of CMVP A144L with TFMK-1 (MW 545) (2), TFMK-2 (MW 465) (3) and DBQ (MW 489) (4), was prepared with molar ratios of 1:5:5:5, respectively. The ESI mass spectrum exhibited peaks with corresponding molar ratios of 1 : 0.15 : 0.083 : 2.17. These results indicate that under the experimental conditions the tightest binding compound to the protease relative to that of the GPC packing material was DBQ.

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Inhibition Mechanisms and Kinetics of Human Cytomegalovirus Protease Inhibitors Analyzed by ESI/MS

Marshall M. Siegel, Keiko Tabei, Geraldine A. Bebernitz, Jeff Holmes, Wei-Dong Ding, Ellen Z. Baum
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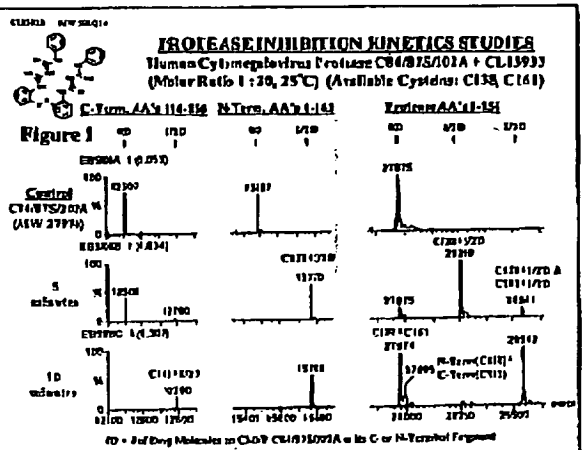
INTRODUCTION Human cytomegalovirus (HCMV) is a virulent pathogen often found in immunocompromised patients. The virus could be suppressed if the HCMV protease associated with it is inhibited. Using random screening methods, a number of chemical agents were found which inhibit the HCMV protease. To better understand the nature of the chemical reactions between the protease and different inhibitors, electrospray ionization mass spectrometry (ESI/MS) was used to both characterize and quantitate reaction products of the protease and inhibitors. Methods were developed which allowed the identification of three distinct mechanisms of inhibition: (I) covalent adduct formation between protease and inhibitor, (II) inhibitor-induced disulfide bond formation within the protease, and, (III) tight binding of the inhibitor to the protease. The kinetics of mechanism I was also monitored so as to provide information on the reaction rates of the different reaction sites of the protease with the inhibitor.

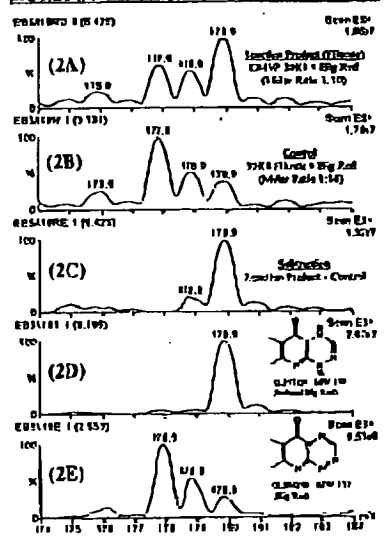
RESULTS The experimental results for the various mechanistic and kinetic studies are summarized below:

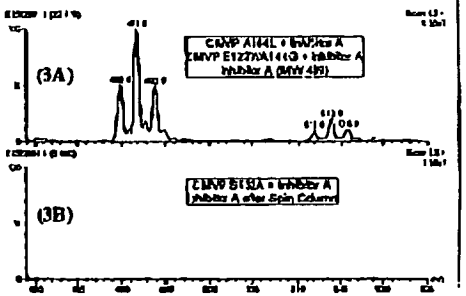
1. *Reaction Kinetics and Inhibition Mechanism Type I: Covalent Adduct Formation Between Protease and Inhibitor and Formation of a Protease Intramolecular Disulfide Bond* Wild type HCMV protease contains five cysteine residues (C84, C87, C138, C161 and C202). Since CL13933 contains a disulfide, the inhibition mechanism was thought to involve disulfide chemistry between the inhibitor and cysteines of the protease. Previous data (Baum *et al*, Biochemistry (1996)) indicated that disulfide bond formation between C138 and C161 inhibits the enzyme. The reaction and kinetics of inhibitor CL13933 with HCMV protease was monitored, using mutated recombinant HCMV protease C84S/C87S/C202A (MW 27,976). This mutant protease contains only the cysteine residues C138 and C161 (the others were mutated and retain enzymatic activity). The first 10 minutes in the reaction between this protease and inhibitor CL13933 (MW 568) is illustrated in Figure 1. Initially, at 0 time, the transformed ESI mass spectrum for the protease exhibits a molecular mass (MW 27,975) consistent with the proposed structure (AA's 1-256) as well as two autodigestion products with MW's 15,487 and 12,507, referred to as the N-terminal fragment (MW 15,487, AA's 1-143) and the C-terminal fragment (MW 12,507, AA's 144-256), respectively. The protease reaction products as well as the autodigestion products were monitored during the reaction. The autodigestion products give structural details for the reaction because the protease reaction sites for the inhibitor are consistent with the only available cysteines at AA's 138 (found in the N-terminal fragment) and 161 (found in the C-terminal fragment). At 5 minutes into the reaction, half of a CL13933 molecule reacted rapidly with the protease (MW 28,259) at C138 while C161 was unreactive. As the reaction proceeded, at 10 minutes, the protease reacted further with a total of one molecule (2 half molecules) of CL13933 (MW 28,542) at sites C138 and C161, the molecule with MW 28,259 disappeared and two forms of the protease appeared (MW's 27,974 and 27,995) consistent with the formation of an intramolecular disulfide linkage within the intact protease (between C138 and C161, MW 27,974) and the disulfide linked complex of the N- and C-terminal autodigestion products (MW 27,995). The difference in mass between these two molecules is 18 daltons, corresponding to the mass of a water molecule. With increasing reaction times, the abundances of these reaction products increase. These ESI/MS results demonstrate that the inhibition mechanism is consistent with covalent disulfide formation within the protease and between the protease and inhibitor.

2. *Inhibition Mechanism Type II: No Covalent Adduct Formation Between Protease and Inhibitor and Formation of an Intramolecular Protease Disulfide Bond* The reaction products of inhibitor CL384188 (MW 177) with HCMV proteases was monitored by ESI/MS. The mass spectral data indicated that no protease-inhibitor conjugate was formed, the protease was oxidized (intramolecular disulfide bonds were formed between C84-C87 and C138-C161 and C202 did not react) and that the inhibitor was reduced (MW 179). ESI/MS data of the HCMV protease A144L (MW 28,083) before and after reaction with CL384188 produced similar spectra consistent with the predicted MW for the protease. However, when the respective materials were further reacted with iodoacetamide (IAM) to determine the number of free cysteines, the observed MW's 28,370 and 28,137 corresponded to the respective addition of 5 and 1 IAM equivalents. (Each IAM equivalent contributes a mass increment of 57 daltons.) The difference in mass between the two samples corresponds to the formation of two disulfide bonds in the protease formed after reaction with CL384188. This oxidation is confirmed by the reduction of CL384188 to CL277439 (MW 179) as indicated in Figure 2. Hence, the analysis of low and high mass products by ESI/MS enabled the elucidation of Mechanism II.



3. *Inhibition Mechanism Type III: Only Non-Covalent Tight Binding Between Inhibitor and Protease* A general method was developed for quantitating the extent of non-covalent binding between a protein (protease) and inhibitor using ESI/MS. The assay is performed in the following manner. The reaction products of the protease and inhibitor are passed through a GPC column, referred to as a spin column, which selectively passes high MW proteins and traps low MW inhibitors. If the inhibitor binds tightly to the protein, both the inhibitor and protein pass through the spin column. ESI/MS is used to assay the effluent and to quantitate the amounts of protein and inhibitor based on known standards. Figure 3 illustrates a qualitative assay demonstrating that the inhibitor does not pass thorough the spin column when alone or in the presence of HCMV protease S132A, but the inhibitor does pass through the spin column in the presence of HCMV proteases A144L and E122V/A144G. This ESI/MS non-covalent binding assay methodology is useful for screening a variety of inhibitors in the presence of different proteases.





DEDICATION This presentation is dedicated to the fond memory of our beloved colleague and friend Yakov "Yasha" Gluzman who inspired us to achieve in our technical fields.